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Invention of

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in

METHODS FOR MODULATING HEMATOPOIESIS AND VASCULAR GROWTH

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APPLICATION FOR UNITED STATES PATENT

METHODS FOR MODULATING HEMATOPOIESIS AND VASCULAR GROWTH

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Cross Reference

This application claims priority from provisional application Serial No. 60/037,513 filed February 10, 1997 and provisional application Serial No. 60/049,763 filed June 16, 1997, both applications being here incorporated by reference.

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Technical Field

Novel methods and compositions are provided, for modulating hematopoeisis and vascular growth *in vitro* and *in vivo*.

Background Art

The life of a new individual is initiated by the fusion of genetic material from the two gametes, the sperm and the egg. After several rounds of division, the cells begin a process of differentiation that ultimately results in the mature adult organism. The process involves many steps including a diverse number of factors which act at specific times during the pathway leading to maturation. The maturation to the adult form does not completely terminate the differentiation process. This is because the adult organism has, in addition to fully differentiated cells, undifferentiated stem cells that are available for both replenishment of differentiated cells during the natural cycle of degeneration and regeneration; and also for repair of damaged tissue. Examples of undifferentiated cells in the adult are bone marrow stem cells (more specifically hematopoeitic stem cells and progenitor cells) as well as endothelial progenitor cells. Cells of this sort provide a therapeutic toolbox in nature for repair and reconstitution of damaged or diseased tissue in a patient. The use of this therapeutic tool box by health care providers for

treating patients is limited by the lack of methods to manipulate the differentiation pathways of these cells and to prepare or boost existing numbers of undifferentiated cells without triggering differentiation.

There is a need therefore to find novel methods in which the supply of undifferentiated cells from any particular individual may be increased, for example, by stimulating the proliferation of the cells without inducing differentiation. It is also desirable to modulate differentiation of undifferentiated cells in a controlled manner. Undifferentiated cells that are ready to differentiate when stimulated to do so offer a treatment to subjects that suffer from diseases in which either the stem cells themselves become depleted such as in chemotherapy which destroys bone marrow, or alternatively for diseases in which differentiated cells are being depleted at a rate that is greater than the body can compensate for the loss by means of using the natural supply of undifferentiated stem cells. For example, in AIDS there is a rapid destruction of mature blood cells by the human immune deficiency virus resulting in a dramatic decrease of immune cells in the patient. There is a need to identify factors that cause stem cells to proliferate and that can modulate differentiation so as to enhance the availability of such cells.

The adult organism contains both endothelial stem cells and hematopoietic stem cells (HSC). These cells are undifferentiated but under appropriate conditions, differentiate to form blood cells and blood vessels respectively. Although there have been extensive studies on vascular growth in the adult, it is unknown whether vascular growth is restricted to vessel extension (angiogenesis) or whether there is *de novo* vascular development (vasculogenesis) also. The understanding of factors that regulate vascular growth is not only important in understanding how to inhibit abnormal vascular growth such as occurs in tumors, rheumatoid arthritis, hemiangiomas, angiofibromas, psoriasis and capillary proliferation and diabetes but also in understanding how to repair vessels after traumatic events including surgery, transplantation and nutrient deprivation to tissues such as occurs in vascular diseases such as cardiovascular or cerebrovascular diseases.

In contrast to vascular growth, hematopoiesis is normally a continuous process throughout the life of an adult. Blood cells are regularly degraded and new cells are formed resulting in a daily production of millions of mature blood cells. Numerous diseases result from

imbalances between degradation and reconstitution of blood cells or from generation of inappropriate numbers of certain blood cells. A simplified schematic of blood cell differentiation is provided in Figure 12. This schematic shows the developmental pathway of eight different types of blood cells that may be derived from a hematopoietic stem cell (HSC) and which passes through an immature progenitor stage. The pluripotent hematopoietic stem cell gives rise to erythrocytes, neutrophils, basophils, eosinophils, platelets, mast cells, monocytes, tissue macrophages, osteoclasts and T and B lymphocytes through a number of different pathways. In the adult, erythrocytes are formed when the pluripotent stem cell differentiates into BFU-E (a burst forming unit-erythroid), which in turn forms a CFU-E (colony forming unit-erythroid). Organs which form blood cells in the adult include bone marrow and to a lesser extent, liver whereas the spleen is the primary site of subsequent clearing of aged or abnormal blood cells. Although the search for factors that regulate hematopoiesis has not been restricted to adults, studies in embryos has been restricted to events that occur when the embryo is already at a relatively advanced stage of development .

With regard to cellular events in the embryo, Cumano et al., *Lymphoid Potential, Probed before Circulation in Mouse, Is Restricted to Caudal Intraembryonic Splanchnopleura*, 86 (1996) 907-16, proposed that the hematopoietic stem cells (HSC) that populate the adult arise from an intraembryonic site. Blood cells reported to first arise in blood islands in the embryo, appear to originate from hematopoietic progenitor cells in the para-aortic splanchnopleura within the developing embryo. (Cumano et al.(1996). The early development of a mouse is shown in Figure 14 and the region of early blood island formation is identified on the periphery of the extracoelomic cavity.

At present, there are a number of growth factors that are known to stimulate early stage intermediate cells in different ~~hematopoietic~~ hematopoietic pathways. These include the hematopoletic growth factors, erythropoietin, granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GMCSF). For example, CFU-E respond to erythropoietin to produce the first recognizable differentiated member of the erythrocyte lineage, the proerythroblast. As blood oxygen levels fall, erythopoietin levels increase, leading to the production of more red blood cells. As a red blood cell matures, it becomes an erythroblast,

synthesizing an enormous amount of hemoglobin and then an erythrocyte. Erythrocytes leave the bone marrow to undertake oxygen delivery to bodily tissues. Although the known factors may have utility in the treatment of certain malignancies or hematologic/immune deficiencies, there is a great need for development of additional therapies, particularly those with a wider range of biological activities that act earlier in the differentiation pathway. The availability of a molecule that could stimulate proliferation and/or differentiation of HSC early in the pathway of differentiation would be especially valuable as a therapeutic. However, there are no factors that are known beyond doubt to stimulate the growth of pluripotent HSC themselves. A protein called stem cell factor has been identified to be associated with pluripotent hematopoietic cells, but this factor is believed to be a survival factor and not a factor capable of stimulating proliferation of these cells (Caceres-Cortes et al., *J. Biol. Chem.*, 269 (1994), 12084-91). There is a need to regulate proliferation and differentiation of hematopoietic stem cells. For example, it would be desirable to inhibit uncontrolled proliferation of stem cells or progenitor cells such as occurs in certain pathological conditions. There is a need for methods to expand the number of pluripotent HSC either *in vitro* or *in vivo* for use in treating patients with chronic anemia or those undergoing chemotherapy where the majority of their bone marrow cells are destroyed so that it is necessary to effectively stimulate the remaining cells or for increasing the availability of HSC for transplantation to an anaemic patient.

Summary of the Invention

This invention satisfies the above need by providing novel methods and compositions that modulate proliferation and/or differentiation of undifferentiated mesodermally derived cells so as to have an effect on at least one of vascular growth and hematopoiesis.

In an embodiment of the invention, a method is provided for stimulating a population of undifferentiated mesodermally derived cells, to undergo at least one of hematopoiesis and vascular growth. The method includes the steps of selecting a compound that is functionally equivalent to a gene product expressed in an embryo's extraembryonic tissue; and causing the compound to access the cells, so as to stimulate the cells to undergo at least one of hematopoiesis and vascular growth.

In another embodiment of the invention, a method is provided for treating developmental errors in vascular growth or hematopoiesis in an embryo *in utero*, that includes the steps of: selecting an effective dose of a compound that is functionally equivalent to a gene product expressed in an extraembryonic tissue; and causing the compound to access a population of embryonic cells *in vivo*, so as to stimulate the cells to undergo at least one of hematopoiesis and vascular growth.

In another embodiment of the invention, a method is provided for treating a subject suffering from an abnormal number of erythroid cells, that includes the steps of selecting an effective dose of a compound that is functionally equivalent to a gene product expressed in an extraembryonic tissue; and causing the compound to access a population of hematopoietic stem cells over an effective time so as to modulate the number of cells undergoing at least one of proliferation or differentiation.

In another embodiment of the invention, a method is provided for treating a subject suffering from an ischemia in tissues containing mesodermally derived cells, that includes selecting an effective dose of a compound that is functionally equivalent to a gene product expressed in an extraembryonic tissue; and administering the compound to the ischemic site over an effective time so as to stimulate vascular growth.

In another embodiment of the invention, an *in vitro* assay is provided for determining the activity of a compound capable of modulating hematopoiesis or vascular growth, that includes the steps of selecting a population of cells from a tissue derived from a fertilized egg of a mammal, wherein the population of cells is deficient in blood formation as detectable by the absence of a predetermined marker; and adding an agent to the population of cells so as to reverse the deficiency.

In another embodiment of the invention, an assay is provided for determining the activity of a compound capable of modulating hematopoiesis or vascular growth, that includes the steps of selecting a first transgenic animal carrying a marker:ϵ-globin hybrid gene, wherein the ϵ-globin gene is capable of expression at least up to 15.5 dpc; mating the first transgenic animal to a second animal that is similarly transgenic; isolating an embryo from the mating at a time within the first third of the gestation period; and determining the effect of the compound on the

stimulation of hematopoiesis and vascular growth in the isolated embryo by measuring marker expression.

Brief Description of the Drawings

The foregoing features of the invention will be more readily understood by reference to the following detailed description taken with the accompanying drawings.

Fig. 1 shows four expression cassettes used to generate transgenic mice. (a) -179 lacZϵμLCR (MB70) is the basic construct with the minimal ϵ-globin promoter (ϵ-pro), extending to -179 with respect to the start site of transcription (+1) and ~20 bp of ϵ-globin 5'-untranslated region (shown as small black box below +1). ϵ-pro is linked to a LacZ expression cassette containing a Kozak consensus sequence and translational start site (SDK region). Downstream from the LacZ reporter gene is a portion of the 3'-region of the ϵ-globin gene containing part of exon 2, all of intron 2 (IVS 2) and all of exon 3; these sequences are shown as black boxes (exons) and a black line (intron). The 3'-untranslated region (containing the polyadenylation site, pA) is shown as a striped line. A truncated version of the LCR (the μLCR) is located downstream from the ϵ-lacZ sequences. (b) -849 lacZϵμLCR (MB73), (c) ϵ-PRE(II+V)lacZϵμLCR (MB72); and (d) -2kblacZϵμLCR (MB92). (a)-(d) contain ϵ-pro and different portions of the upstream regulatory region of the human ϵ-globin gene. Eukaryotic sequences in (a)-(d) were excised from the vector by digestion with KpnI and NotI and then purified for microinjection into the male pronuclei of mouse zygotes.

Fig. 2 shows LacZ expression, correlated with the appearance of a blue stain, in primitive erythroblasts. A (a) is a diagramatic representation of a 7.5 dpc embryo; (b) is a transgenic embryo stained with XGal and viewed by bright field microscopy, (c) is the same embryo viewed by dark field microscopy; B (a) is a non-transgenic mouse; (b) and (c) are embryos at 8.5 dpc stained with XGal; C(a) is a wild type 12.5 dpc embryo, (b) and (c) are transgenic 12.5 dpc embryos. (1) wild type; (2) transgenic; (3) ectoplacental cone; (4) blood islands; (5) amniotic cavity; (6) trophectoderm; (7) allantois; (8) extraembryonic mesoderm; (9) embryo proper (epiblast).

Fig. 3 shows the formation of yolk sac-like structures by cultured blastocysts (a) transgenic blastocysts prior to culture (b) Sac-like structure (non transgenic) stained with benzidine to reveal hemoglobin containing cells (c) Sac from cultured transgenic blastocysts stained with XGal to reveal hemoglobin containing cells after 9 days of cultivation (d) Normal 8.5 dpc transgenic embryo and yolk sac stained with XGal.

Fig. 4 shows RT-PCR analysis of blastocyst cultures: ε-globin was observed in blastocysts that have developed into sac-like structures (sac) but not in samples that were relatively flat mounds of cells (flat). The higher molecular weight band is the internal control-actin. The lower molecular weight band is embryonic β-globin.

Fig. 5 shows that primitive erythropoiesis initiates late in gastrulation. On the left is a whole-mount *in situ* analysis of late-streak (~7.5 dpc) embryo and on the right is an early streak (~6.5 dpc) embryo. The purple stain is a chromogenic substrate. The ε-globin RNA probe reveals hemopoietic cells in the embryo on the left as shown in the rostral (anterior) view, and none in the embryo on the right demonstrating absence of blood formation in the 6.5 dpc embryo.

Fig. 6 shows differential expression of *patched* (ptc) and *Gli* genes in dissected yolk sac mesoderm at 10.5 dpc and 12.5 dpc by means of RT-PCR analyses, revealing substantially exclusive expression of *Gli* and *ptc* in dissected yolk sac mesoderm: whole, undissected yolk sacs; meso, mesoderm layer; endo, endoderm layer; -cDNA, minus cDNA control. Actin served as an internal control.

Fig. 7 shows that when transgenic explants of gastrulating embryos (isolated at 6.25-6.5dpc) are cultured on filters or glass slides for 72 hours, induction of embryonic hematopoiesis occurs in whole embryo, but is absent in epiblasts only, as determined by XGal staining. Dashed lines were drawn around the epiblasts to facilitate visualization of structures. (a) whole embryo on a filter; (b) epiblast on a filter; (c) whole embryo on a slide; and (d) epiblast on a slide.

Fig 7-1 shows blood formation in transgenic embryonic explant cultures. (lacZ stained sections of embryos). Frozen tissue sections were XGAL stained to reveal cluster of lacZ-positive hematopietic cells in the whole embryos (a), epiblasts (b), posterior embryo portions (c,d) and transgenic anterior epiblast portion adjacent to the VE (e). but not in surrounding visceral endoderm and undifferentiated mesoderm nor in the nontransgenic VE tissue of anterior/VE recombinants(e)

Fig. 8-1 shows induction of hematopoiesis by visceral endoderm (VE) signals. (a) dark-field photomicrograph of recombinant containing transgenic (Tg) epiblast and non-Tg VE showing localized lac Z staining in the embryo adjacent to the visceral endoderm; (b) schematic diagram corresponding to panel (a). Abbreviations: Tg , transgenic; Ve, visceral endoderm; EryP, primitive erythroid cells; (c) bright field photomicrograph of recombinant shown in (a).

Fig. 8-2 shows induction of embryonic hematopoiesis in whole embryo, and in epiblast plus visceral endoderm, but none in epiblasts only, using RT-PCR. (All samples were prepared following a 72 hour *in vitro* incubation of embryos isolated at 6.5 dpc). Actin served as an internal control.

Fig. 9 shows that recombinant hedgehog protein can substitute for visceral endoderm to stimulate primitive hematopoiesis in cultured epiblasts. Isolated epiblasts were cultured in the absence (lanes labeled "none") or presence of three different concentrations of recombinant hedgehog protein (0.25, 1 and 5 µg/ml). Primitive hematopoiesis was assessed by RT-PCR analysis for ε-globin expression. Actin served as an internal control. YS, yolk sac control.

Fig. 10 shows the activation of primitive erythropoiesis by a diffusible factor in visceral endoderm cells by means of RT-PCR analysis.

Fig. 11 shows the inhibition of primitive erythropoiesis in cultured whole embryos using a SHH blocking antibody by means of RT-PCR analysis.

Fig. 12 shows a schematic representation of the adult hematopoietic hierarchy.

Fig. 13 shows the derivation of cell lineages in the mammalian embryonic yolk sac. The circular structure represents a blastocyst of around 3.5 days.

Fig. 14 shows the early development of the mouse. The region of early blood island formation occurs in the exocoelomic cavity (f) between the epiblast below which is surrounded by the visceral endoderm and the extraembryonic tissue above.

Fig. 15 shows the experimental scheme for separation of epiblast into anterior and posterior portions. (A) depicts the entire 6.75 dpc embryo with visceral endoderm around the perimeter of the epiblast and the extraembryonic mesoderm. (B) depicts the embryo after the visceral endoderm has been stripped off and (C) shows the epiblast only, with a dotted line of transection showing how the anterior and posterior sections are physically divided before separate cultivation.

Fig. 16-1 shows that hematopoietic mesoderm arises from the posterior primitive streak (posterior mesoderm) when anterior and posterior portions of lacZ transgenic embryos are harvested at mid- to late-gastrulation. Panel A: No staining is detected in anterior epiblasts. Panel B: Dark blue XGAL histochemical staining shows blood formation in cultured posterior epiblasts. Scale bar, 1mm.

Fig. 16-2 shows that visceral endoderm can reprogram the anterior embryonic ectoderm (epiblast) to express hematopoietic markers. The expression of ϵ-globin, β-globin, GATA-1, and CD34 markers is shown for anterior epiblast (anterior: lanes 6-10), posterior epiblast (posterior: lanes 6-10) and anterior recombined with visceral endoderm (a/Ve recombs: lanes 1-5). Control tissues were uncultured whole embryo [emb(-cx)], cultured whole embryos[emb(+cx)] and 10.5dpc yolk sac tissue. The control marker was actin and cardiac myosin. An additional control is emb(+cx) subjected to PCR in the absence of reverse transcriptase.

Fig. 16-3 Visceral endoderm can reprogram the anterior embryonic ectoderm (epiblast) to express vascular markers. The expression of PECAM-1, flk-1 and actin is shown for anterior epiblast (anterior: lanes 6-10), posterior epiblast (posterior: lanes 6-10) and anterior recombined with visceral endoderm (a/Ve recombs: lanes 1-5). Control tissues were uncultured whole embryos [emb(-cx)], cultured whole embryos [emb(+cx)] and 10.5 dpc yolk sac tissue. The control marker was actin.

Fig 17 shows the results of a rescue experiment using null mutant embryonic stem cells (ES) and adding back recombinant BMP-4 to the culture. (A) and (C) shows wild type embryoid

bodies that arise from embryonic stem cells isolated from a wild type mouse. In (B) the embryonic stem cells are homozygous BMP-4 ~~deficient~~ deficient and the embryoid bodies lack detectable blood formation. In (D), BMP-4 protein is added to the embryoid bodies of (B) and blood formation is observed.

Detailed Description of the Invention

The invention identifies for the first time methods for stimulating selected developmental activities in embryonic and adult tissue namely blood development characterized by hematopoiesis and vascular growth. The method further utilizes molecules secreted by extra-embryonic tissues capable of modulating the proliferation or differentiation of stem cells and progenitor cells from embryo or adult . Embodiments of the invention are further directed to novel assays for identifying compounds capable of stimulating hematopoiesis and vascular growth. Support for the methods of the invention are provided in the examples contained herein. According to an embodiment of the invention, compounds have been identified that are capable of stimulating blood development in the embryo and in the adult and are functionally equivalent to gene products expressed in the visceral endoderm and yolk sac mesoderm. Such gene products are exemplified by hedgehog compounds, TGF-β, TNF , and WNT compounds and are here identified as achieving a similar effect to that observed with extraembryonic tissues with regard to hematopoiesis and vascular growth in undifferentiated mesodermal derived tissues. In an embodiment of the invention, compounds including those selected from hedgehog and TGF-β may act synergistically so as to enhance their stimulatory effect on target cells.

"Adult" is defined here and in the claims as descriptive of tissues and cells derived from or within an animal subject at any time after birth.

"Embryonic" is defined here and in the claims unless stated otherwise as descriptive of tissues and cells derived from or within an animal subject at any time prior to birth.

"Blood development" is defined here and in the claims as hematopoiesis and vascular growth.

"Vascular growth" is defined here as at least one of vasculogenesis and angiogenesis and includes formation of capillaries, arteries, veins or lymphatic vessels.

"Hematopoiesis" is defined here and in the claims as the process of production of blood cells.

"Hematopoietic stem cell" is defined here and in the claims as a multipotential precursor from which all classes of blood cell are derived.

"Definitive blood cells" are defined here and in the claims as blood cells of the fetal or adult organism.

"Primitive blood cells" are defined here and in the claims as a transient population of blood cells forming during blood development in the embryo.

"Definitive endoderm" is endoderm that is derived during gastrulation from cells of the primitive streak that contribute to adult endodermally derived tissues such as gut and liver.

"Visceral endoderm" is defined here and in the claims as extraembryonic endodermal cells that are secretory and do not contribute directly to any tissues of the fully formed organism.

"Progenitor cells" are defined here and in the claims as undifferentiated cells that are more restricted in their potential to give rise to differentiated cell types compared with a stem cell.

"Committed" is defined here and in the claims as cells destined to differentiate along a specific lineage instead of retaining multipotency.

"RT-PCR" is defined here as reverse transcriptase polymerase chain reaction which permits detection of transcription of a gene in a tissue.

"Synergistic effect" is defined here as for two or more compounds where little or no biological effect is observed with the compounds alone but together the compounds have a potent biological effect.

"Hedgehog compound" is defined here and in the claims as a class of molecules of the hedgehog family that includes recombinant hedgehog protein, analogs, and derivatives of hedgehog proteins, and agonists and antagonists of hedgehog protein receptors and functional equivalents of the aforementioned.

"Undifferentiated mesodermally derived cells" is meant here and in the claims to include cells that are undifferentiated or uncommitted and further to include stem cells and progenitor cells

CFU-E is here defined as erythroid colony-forming cell (unit),which is a late (mature) erythroid progenitor cell. Colonies scored as CFU-E are small, tight clusters of pigmented cells and appear within 2-3 days of culture.

BFU-E is here defined as erythroid burst-forming unit, a primitive erythroid cell. These colonies are pigmented and larger in size than CFU-E; their cells are more widely dispersed, and they appeared at a later time after plating. Their numbers are maximal around 7 days in culture.

CFU-GM is here defined as myeloid or granulocyte-macrophage colony forming unit. These are similar to BFU-E in appearance but are unpigmented.

CFU-S is here defined as spleen colony-forming unit.

Hematopoiesis and vascular growth are some of the first requirements of a growing tissue mass to secure a supply of nutrients to cells in the interior of the mass. The developing embryo requires nutrition and, therefore, the differentiation of cells to form erythroblasts (oxygen carrying cells) and the formation of a vascular system (transport system) are one of the first events in the developmental process. While the embryonic tissue is undergoing cell movement reminiscent of that seen in avian and reptilian gastrulation (migration of mesoderm and definitive endoderm cells through a primitive streak), the extraembryonic cells are making mammalian tissue that enable the fetus to survive in the maternal uterus. This includes stimulating a maternal blood supply to form the uterine endometrium. The narrow connecting stalk of extraembryonic mesoderm that links the embryo to the trophoblast eventually forms the vessels of the umbilical cord. The fully developed organ, consisting of trophoblast tissue and blood vessel-containing mesoderm, is called the chorion. The fusion of the chorion and the uterine wall forms the placenta. By 4 weeks post-conception, the human embryo has a source of nutrients through fetal blood vessels that are adjacent to the maternal circulation.

In the adult, vascular growth occurs during the repair of damaged tissues and in a variety of diseases including cancer, where a tumor releases factors that stimulate sprouting of blood vessels in normal tissue where the new blood vessels are directed into the tumor tissue. The hematopoietic stem cells (HSC) that populate the adult may arise at an intraembryonic site. (Cumano, et al. (1996)). It is believed that this mesodermal tissue is an important if not major

site of origin of definitive hematopoietic stem cells and perhaps cells that give rise to the vasculature.

Although the process of hematopoiesis and vascular growth is only partly understood, the pathway of development in mice appears to mimic the equivalent process in humans. The mouse hematopoietic system is derived from the mesodermal germ layer which begins to form in primitive-streak-stage embryos around 6.5 dpc. Blood islands first appear in the extraembryonic mesoderm at 7.5 dpc and hematopoietic progenitors in the visceral yolk sac mesoderm of embryos within 1-2 somite pairs at 8 dpc. At mid-8 dpc, nucleated primitive red blood cells are visible in the vasculature of the yolk sac but do not enter the primitive circulation system until 8.5 dpc. Beginning at 8.5 - 9 dpc, hematopoietic progenitor cells have been found in mesodermally derived regions within the embryo body, notably the para-aortic splanchnopleura (Cumano, et al.(1996). Splanchnic mesodermal cells lining the yolk sac form cords of cells that hollow into a tube lined by endothelial cells. The central cells of the blood islands differentiate into embryonic blood cells. As the blood islands grow, they eventually merge to form the capillary network and the vitelline vessels that ultimately connect to the newly formed heart.

Until now, little has been known about the biochemical events prior to about 8dpc that play a role in vasculogenesis and hematopoiesis. However, we assert that this stage in development play a significant role in the maturation of the blood system in the embryo and in the adult. According to embodiments of the invention, processes of vascular growth and hematopoiesis in embryonic development are affected by compounds in the visceral endoderm. For example, we have identified for the first time that hedgehog proteins act on undifferentiated mesodermal derived cells *in vitro* to stimulate blood formation and on embryonic tissue and yolk sac development at very early stages in the hematopoiesis and vascular growth pathways. Furthermore, according to the invention, these early acting compounds have utility in regulating hematopoiesis and vascular growth in the adult animal. (Table 1 and 2). According to embodiments of the invention, "stimulating a population of undifferentiated mesodermally derived cells to undergo at least one of hematopoiesis and vascular growth" includes stimulating proliferation of hematopoietic stem cells and progenitor cells prior to differentiation (Example 4)

The identification of factors in visceral endoderm that stimulate blood development and vascular growth is here demonstrated through the use of novel assays. These assays include:

(a) the analysis of embryonic explant tissues prior, during and after blood development. For example, explants may be derived from blastocysts which are formed at the first stage of mammalian development. Blastocysts are formed when the embryo reaches the 64 cell stage forming an inner cell mass, an outer trophoblast cell layer formed from trophectoderm cells and an internal space containing fluid identified as the blastocoel. The inner cell mass (ICM) is situated in the blastocoel and becomes segregated into the "primitive endoderm" which forms the outer layer of the ICM, and the ICM itself. The "primitive endoderm" give rise to parietal and visceral endoderm. The internal ICM cells, which form the primitive ectoderm, gives rise to the embryo proper. Blastocysts, which are isolated before blood development is initiated, can be maintained in culture for periods of time that allow for the formation of tissues characteristic of organs associated with the vasculature such as beating cardiac muscle. Blood development is first observed histologically when blood islands are observed between the endoderm and the mesoderm of the developing embryonic yolk sac. Without being bound by theory, we believe that the observed islands are formed as a result of the formation of erythroblasts and endothelial cells from undifferentiated precursor cells. In the experimental mouse model, embryonic explants may be isolated at or after 2 dpc before blood island formation is observed and may be maintained *in vitro* up to 14 dpc and longer when organ development is in progress, providing a model system for following the initiation and progression of blood and the vascular system. Isolation of explants prior to formation of blood according to the invention is novel with respect to the prior art, where the prior art describes events in blood development after initiation of blood island formation has already occurred. (Cumano et al. (1996), Palis et al., *Blood* 86 (1995), 160-63, Kanatsu et al., *Development* 122 (1996), 23-30).

(b) The use of explants from transgenic animals in which the regulatory region of an early stage gene product associated with blood formation (for example ϵ-globin gene) is coupled to a marker, such that the marker (for example LacZ) serves to signal the onset of hematopoiesis and vascular growth [Example 1]. Onset of hematopoiesis or vascular growth can be detected using sensitive detection methods such RT-PCR which can detect initiation of expression and

the extent of expression of gene products that are associated with blood development; histochemical staining exemplified by benzidine staining of hemoglobin; immunohistochemistry that utilizes an antibody of appropriate specificity; whole mount *in situ* hybridization; *in situ* hybridization using radiolabelled riboprobes and other detection methods known in the art.

Four different assay designs are described below and in Examples 1 and 2 which have utility both individually and in combination for screening and identifying factors involved in hematopoiesis and vascular growth.

(i) Epiblast cultures: Intact embryo explants were harvested prior to the histological appearance of blood islands for example at 6.5dpc, and incubated intact *in vitro* using standard culture techniques so as to permit development of the embryo to continue thereby serving as positive controls of blood formation.(Example 2A-B). In these circumstances, the explants formed blood islands *in vitro* and blood formation could be followed by measuring the appearance of markers of early blood development such as ϵ-globin,(embryonic β-globin) GATA-1, CD 34, sca-1 (markers of hematopoietic stem cells) PECAM-1, flk-1, Vezf-1 (endothelial marker). In Example 2, ϵ-globin gene expression was detected after a 72 hr incubation *in vitro* in epiblast cultures using RT-PCR. Embryos explanted from transgenic animals, carrying the LacZ marker under the ϵ-globin gene promoter, stained with XGal after a similar time period.

The assay includes the creation of embryo explants that are not capable of producing blood islands, where this incapacity is reversible. When embryos were first stripped of the visceral endoderm, the resulting epiblasts failed to stain positively with XGal in explant cultures and therefore failed to express ϵ-globin (Figs. 7, 8). Diffusible factors have been identified that modulate the initiation of hematopoiesis in a manner independent of direct cell-cell contact. These factors are made outside of the epiblast, for example, in the visceral endoderm. The biological role of these factors was confirmed by reconstitution experiments using visceral endoderm and epiblast tissues (Figure 8). The requirement for factors contained in the visceral endoderm was further demonstrated when we compared the effect of treating epiblasts with conditioned medium obtained from visceral endoderm cell cultures compared with control untreated epiblasts (Example 2A). Whereas, in the absence of conditioned medium, epiblasts did

not express ϵ-globin, the addition of conditioned medium containing secreted cell factors induced the expression of ϵ-globin in the embryonic tissue (Fig. 10). Epiblasts derived from non-transgenic mice were analyzed for gene expression using whole- mount *in situ* hybridization or immunostaining.

(ii) Blastocyst cultures: The expression of XGal in blastocysts derived from transgenic mice containing a hybrid gene formed from a detectable marker controlled by regulatory sequences of a gene associated with blood formation such as embryonic β-like: LacZ globin (ϵ-globin: LacZ) was measured. In Example 2(C), blastocysts were isolated at 3.5dpc and incubated for a further 7-10 days. (Figure 13) Alternatively, blastocyst cultures have been prepared as above using non-transgenic mice and gene expression has been detected by whole mount *in situ* hybridization or immunostaining. Details of blastocyst cultures are provided in Example 2(C) and in Figure 3.

(iii) Modified epiblast culture assays: Late stage gastrulating embryos have been harvested and epiblasts prepared by dissecting away the extraembryonic ectoderm (Example 2(B)). During gastrulation, embryonic cells establish the basic body plan and extraembryonic mesoderm contribute to the extraembryonic tissues, respectively. Mesoderm cells destined for extraembryonic sites exit the posterior primitive streak and subdivide the embryo into three separate cavities by the late streak stage at 7.5 dpc. The central cavity, the exocoelom, becomes completely lined with mesoderm cells. These mesoderm cells lie adjacent to the embryonic ectoderm to form the amnion, the extraembryonic ectoderm to form the chorion and the visceral endoderm to form the visceral yolk sac (VYS). At the end of gastrulation, the cells in the embryo have separated into three germ layers: the outer ectoderm, giving rise to the epidermis and the nervous system; the inner endoderm, giving rise to the lining of the digestive tube and its associated organs (such as pancreas, liver, and spleen); and the intermediate mesoderm, giving rise to several organs (heart, kidney, gonads), connective tissue (bone, muscle, tendons) and the definitive blood cells.

Single epiblasts from late stage gastrulating embryos were transected into anterior and posterior portions and each portion was cultured individually for several days (Figures 14 and 15). While the anterior epiblast portion formed little or no blood islands as determined by

expression of ϵ-globin using RT-PCR techniques, the posterior portions formed blood at levels comparable to the intact epiblast. Using this assay, compositions may be added to the anterior epiblast and the stimulation of blood formation determined. The control in this assay is the addition of visceral endoderm which is sufficient to cause the anterior epiblast to form blood islands. When either visceral endoderm or hedgehog protein was added to the culture, blood formation was observed. (Figure 16)

(iv) Explants or embryoid bodies derived from mutants defective in targeted protein: Embryoid bodies are formed from harvested embryonic stem cells that are incubated *in vitro* using techniques well known in the art.(Example 2(C)) These cells form embryoid bodies that contain several cell types including blood cells and endothelial precursor cells (see Figure 17 (A, C). Embryonic stem cells may be subjected to targeted mutations in selected mouse genes, which products play a role in hematopoiesis and vascular growth, using well established techniques such as homologous recombination and selectable drug resistance, resulting in cells that are homozygous for the targeted gene mutation. Mutations may be induced that (i) "knock out" a coding gene or regulatory sequence ; (ii) "knock out" a coding gene or regulatory sequence and replace the sequence with a "knock-in" sequence that causes something else to be made (the knock-in sequence may be a mutated sequence); or (iii) generate a random mutation by insertion of foreign DNA into the genome or use of chemicals to cause mutations. The consequences of forming such mutations include: modifying the activity of a particular gene product and nullifying the activity of the gene product and may further include substitution of a gene product with another gene product by established methods of genetic manipulation.

Where these targeted mutations result in no gene expression of the protein, the mutations are called null mutations. Null mutants were formed using wild type embryonic stem cells. According to the invention, null mutants defective in a protein that is associated with the visceral endoderm such that its absence results in the failure to make blood, is a suitable model system for screening novel compounds from libraries such as those derived from extraembryonic tissues, where these libraries include combinatorial peptide libraries and recombinant DNA libraries. By using a pooling strategy to reduce the number of experimental tests, compounds may be identified that are useful in modulating hematopoiesis and vascular growth in embryoid bodies.

This general type of assay can be used to study the effect of other mutations, such as deficiency of signaling factors such as hedgehog proteins (for example, Indian hedgehog), on blood formation.(Examples 3-5) For example, *Ihh* null mutant ES cells may be formed and factors capable of overcoming the mutation, identified. These cells could be rescued either by providing exogenous hedgehog protein or by transfecting the cells with vectors expressing a hedgehog gene utilizing standard vectors or retroviral vectors.(Figure 9) The mutated cells could also be reintroduced into mice to form chimeras.

These detection techniques were used to detect hematopoiesis and vascular growth in epiblasts and blastocyst cultures as follows: According to the assay of the invention, the onset of blood island formation may be detected using any of the sensitive techniques available in the art, including the following:

(1) Detection of XGal in explants derived from transgenic mice that contained the hybrid gene -embryonic β-like: LacZ globin (ϵ-globin: LacZ). The embryos of homozygous transgenic mice were analyzed using XGal to reveal globin gene transcription indicative of blood development prior to visual detection of erythroid cells (Figure 1, 2)

(2) Detection of globin gene expression using radioactive semi-quantitative RT-PCR probes in epiblasts and blastocysts at various times post conception. A distinct advantage of using a radioactive assay is that the amount of tissue recovered from individual explants is very small, and a sensitive assay makes it possible to assay for expression of many genes from a single culture product.(Figure 4)

Using the above assays, we have identified a number of compounds that are functionally equivalent to gene products that are expressed in extraembryonic tissues and may stimulate blood formation. These compounds include TGF-β proteins more specifically TGF-β1 more specifically bone morphogenic protein (BMP) more specifically BMP-4; tumor necrosis factor (TNF) proteins more specifically TNF-α; wnt family; and hedgehog proteins. (Figures 5,9 and 17) Compounds may also include naturally occurring and synthetic agonists, antagonists, analogs and derivatives of the above. These molecules may interact with membrane proteins which initiate signal transduction pathways resulting in a biological response. Therefore, in addition to the above compounds, agonists and antagonists to these membrane binding proteins including

those receptors, receptor agonists and receptor antagonists associated with hedgehog binding receptors and hedgehog signalling transduction pathways such as smoothened, patched and gli may have utility in regulating hematopoiesis and vascular growth.

The target site for stimulating stem cell proliferation and modulating differentiation is here identified as predifferentiated mesodermal derived tissue such as is present in the embryo. Embryonic predifferentiated mesodermal tissue includes visceral yolk sac, allantois, amnion, chorion, trophectoderm and prenatal yolk sac, hematopoietic stem cells in fetal liver and umbilical cord blood. Predifferentiated mesodermal derived tissue in the adult includes hematopoietic stem cells and progenitor cells in adult bone marrow, liver and spleen and endothelial stem cells and progenitor cells in the fetus and adult.

The novel assays of the invention are capable of use in multiple applications, including:

(i) screening libraries of compounds for activity in stimulating hematopoiesis and vascular growth;

(ii) testing for the effect of growth factors, cytokines and other signaling molecules on embryonic hematopoiesis and also on vascular growth;

(iii) determining the effect of hedgehog proteins on hematopoiesis and vascular growth in the embryo, fetus and adult. For example, the blastocyst assay may be used to determine the effect of hedgehog proteins on yolk sac development *ex vivo* where the blastocyst is derived from transgenic or non-transgenic animals.

(iv) examining the hematopoietic potential of other embryonic tissues such as the allantois which does not normally produce blood cells but whose mesoderm is of the same origin as that of the yolk sac;

(v) following the development of primitive erythroid cells and vascular structures by staining with a marker such as XGal so as to outline the vasculature and permit the tracking of vascular growth as well as hematopoiesis; and provides the means for analyzing early intraembryonic definitive hematopoiesis as well as primitive yolk sac hematopoiesis;

(vi) determining the effect on individual explants of targeted mutations in genes that affect hematopoiesis or vascular growth in the parent animal including those carrying transgenes expressing hedgehog, patched, Gli and other proteins; and

(vii) examining the effect of gene therapy on mesodermally derived tissues; where for example, the gene for hedgehog protein is introduced into prestreak embryos deprived of the visceral endoderm, under various promoters so as to modulate the effect of blood island formation. This type of gene therapy model may serve as an experimental tool for identifying molecules capable of modulating hematopoiesis and vascular growth.

The newly identified role of morphogenic proteins in hematopoiesis and vascular growth:

Hedgehog proteins: We have shown here for the first time that hedgehog proteins are capable of stimulating hematopoiesis in the yolk sac, and the splanchnopleura and other hematopoietic tissues of the embryo or fetus and of stimulating hematopoiesis in the bone marrow of the adult.(Examples 3-5, Tables 1-2, Figs 6,9). By screening for molecules that were present in the visceral endoderm, we identified hedgehog gene product. When a hedgehog protein (SHH) was added to epiblast cultures and RNA was isolated after 2-3 days and analyzed by RT-PCR (Example 3, Fig. 9), hematopoiesis was observed to be stimulated, as determined by the activation of the ϵ-globin gene. Furthermore, SHH protein was capable of stimulating hematopoiesis in the epiblast absent the visceral endoderm. When antibodies to SHH were added to whole embryos, as described in Example 4, and Fig.11, ϵ-globin expression was substantially reduced.

The above assays show that hedgehog proteins expressed in extraembryonic tissue as well as hedgehog proteins that are closely related to proteins expressed in extraembryonic tissues, stimulate hematopoiesis and vasculogenesis. Members of the hedgehog family which are a distinct family of signaling molecules (e.g., reviewed in Goodrich et al.,*Genes & Develop.* 10 (1996), 301-12) are known to play a role in limb morphogenesis, neural development, bone modeling and spermatogenesis. The family was initially identified as involved in normal segmental patterning in Drosophila (Nusslein-Volhard et al, *Nature,* 287 (1980), 795-801). The hedgehog family includes Desert hedgehog (DHH) protein, Indian hedgehog protein (IHH), Moonrat hedgehog (Zebrafish) and Tiggy winkle hedgehog (Zebra fish).

Although the invention is not intended to be limited by theories, we suggest that the initial expression of IHH in visceral endoderm may result in activation of DHH later in the yolk

sac mesoderm and that DHH may act.on the extraembryonic mesoderm of the yolk sac in an autocrine manner. In this way, epiblasts stripped of visceral endoderm at 6.5dpc may produce blood islands at 7.5dpc in the presence of IHH acting on DHH signaling. Once DHH signaling is initiated in this way, IHH may no longer be absolutely required. We have observed the effect of IHH knockout or DHH knockout alone or together. We note that the DHH knock-out does not prevent the formation of blood islands and conclude that IHH has a continued stimulating effect on blood development in the absence of DHH. We suggest that both IHH and DHH would need to be knocked out to result in a yolk sac phenotype lacking blood cells and vasculature. The apparent functional differences in the molecules themselves may not reside so much in their biochemical differences but rather may follow from differences in the site of expression or the timing of expression. A precedent for this is provided by the engrailed genes (Hanks, et al., *Science,* 269 (1995), 679-82). The propositions presented above represent the preferred explanations for the relationship of DHH and IHH but are not meant to exclude other explanations for the observed associations between these proteins.

The utility of the hedgehog proteins in stimulating hematopoiesis and vascular growth is further reinforced by our experiments on target molecules through which these proteins act. Using RT-PCR to analyze expression of *patched* and *Gli*, (Example 5, Fig. 6) we identified substantially exclusive expression of these proteins in the yolk sac mesoderm, a tissue whose sole function is to produce blood and vascular endothelial cells.

In support of our observations that hedgehog proteins are capable of stimulating hematopoiesis, we identified the enriched expression of *Gli* and *patched* in yolk sac mesoderm. Gli is a transcription factor involved in the transduction pathway on which hedgehog proteins act, while PTC (patched) is a membrane protein that binds hedgehog protein to initiate the signal transduction pathway that ultimately causes a biological response in the target cell. The association of these proteins with yolk sac mesoderm further supports the observation that hedgehog proteins stimulate hematopoiesis. Since *ptc* is the presumed gateway to a cell response, any agonist of hedgehog capable of binding patch is expected to induce the same biological effect as hedgehog-in this case, hematopoiesis and vascular growth.

Certain hedgehog proteins have been reported to be involved in the initiation of expression of the secondary signaling molecules-BMP-2 and BMP-4 (proteins belonging to the TGF-β family) in the mesoderm and Fgf-4 in the ectoderm (WO 95/18856). We have identified for the first time, that hedgehog proteins might interact in a synergistic manner with secondary signaling molecules to stimulate hematopoiesis and vascular growth (Example 6). These signaling molecules include BMP-2, BMP-4 , BMP-6 and BMP-7 and other members of the TGF-β family including Wnts and FGF, which may be found to be associated with the visceral endoderm and/or the yolk sac mesoderm.

The activity of compounds that are functional equivalents to a gene product expressed in extra-embryonic tissue such as recombinant hedgehog protein, analogs, derivatives and dissociation products of hedgehog proteins, and agonists of hedgehog protein receptors such as PTC according to the invention, may stimulate hematopoiesis and vascular growth by acting on cells or tissues from embryos of different ages including fetal cells, fetal peripheral blood and cord blood, as well as on adult hematopoietic stem cells and adult progenitor cells. The invention includes the use of functional peptides of hedgehog protein. The term "functional peptide" as a subclass of a hedgehog compound defined above, is meant to include peptide fragments of the hedgehog protein that are capable of inducing a biological activity that is the same or equivalent to the entire protein (WO 96/16668, ~~incorporated here by reference~~). The invention further includes hedgehog compounds described in WO 95/18856 and here ~~incorporated by reference~~, including homologs of hedgehog proteins, recombinant hedgehog proteins, hedgehog encoding nucleic acids, antisense molecules, gene constructs for use in gene therapy including viral vectors known in the art, combinatorial mutants of hedgehog proteins as agonists or antagonists, and antibodies specific for hedgehog protein epitope. These and other compounds may be selected for modulating hematopoiesis and vascular growth according to the assays of the invention.

According to the invention, these factors may be used to stimulate hematopoiesis and vascular growth in animals including mammals, including humans. Similarly antagonists to the compounds of the invention may be used to inhibit vascular growth and hematopoiesis. The therapeutic utility of these factors is discussed below.

Our novel blastocyst assay may be used to determine the effect of hedgehog proteins on yolk sac development. In addition, blastosacs could be assayed for gene expression not only using LacZ as a histochemical marker, but also by whole-mount *in situ* hybridization or by immunostaining.

Transgenic mouse models for studying the effect of selected compounds on hematopoiesis and vascular growth:

Transgenic mouse models have utility in the study of developmental events. When a histological marker gene is introduced into the genome of mice, patterning associated with marked cells can be established.

The transgenic mice of the prior art have at least four major limitations: (i) the ability to follow transcription of the transgene relies on RNase protection or S1 nuclease assays of mRNA production, and tissue samples may be limiting at earlier developmental stages; (ii) the specificity of expression cannot be examined at the single cell level (short of performing *in situ* hybridizations using riboprobes, but these experiments are technically challenging and expensive); (iii) unbalanced expression of an exogenous β-globin gene in the absence of a counterbalancing α-globin gene is likely to lead to severe thalassemia (Hanscombe, et al., *Genes & Develop.* 3 (1989), 1572-81) and is thought to reduce the yield of transgenic progeny through early death *in utero* (Hanscombe et al., 1989; Pondel et al. *Nucleic Acids Res.* 20 (1992), 5655-60.). (iv) use of the entire globin gene with its upstream regulatory sequences, in transgenic mice and examination of mice after 8.5-9.5dpc has resulted in analysis on blood development post initiation, Pondel, et al., (1992).

We have developed transgenic animals that provide models for blood development that overcame the limitations of the transgenic mice of the prior art. We have here adopted the use of selected mice models in which a marker gene is placed under the control of globin regulatory sequences to obtain explants suited to the assay as described above. We selected a marker, exemplified by β-galactosidase (LacZ) reporter gene, in place of the body of the ϵ-globin gene, so that functional exogenous hemoglobin protein would not be made and so that a sensitive enzymatic assay could be used to follow transgene expression. (Example 1) An additional

advantage of using a reporter gene such as LacZ is that it allows for rapid, detailed histochemical studies in which the specificity of expression can be analyzed at the single cell level or quantitatively in tissue lysates. The ability to examine expression of single cells within a complex tissue is particularly useful for studies involving early embryogenesis.

Alternative reporter genes to that of LacZ include alkaline phosphatase and green fluorescent protein or its derivatives. Embryos formed according to Example 1 may express LacZ at a peak level as early as 7.5 dpc of development, continuing to as late as 16.5 dpc. The LacZ expression in the mouse model of the present invention may be identified in the intraembryonic para-aorta splanchnopleura and in the aorta-gonad-mesonephros (AGM) region; see below). As such, they are uniquely suited for studies on hematopoiesis at later developmental stages and have utility in a variety of *in vitro* and *in vivo* studies on embryonic hematopoiesis. Consequently, these animals have utility as a source of genetically marked erythroid cells for various kinds of explant or embryo cultures.

Using the transgenic methodology described in the invention, LacZ transgenic mice may be used as models for modulation of expression of hematopoiesis and vascular growth in either embryonic or adult animal by utilizing enhancers and/or promoters that direct the timing of expression during development or directing the tissue specificity of expression, such enhancer optionally being inducible. Examples include α-fetoprotein enhancer that directs gene expression to the yolk sac and developing gut, cardiac actin enhancer that directs expression to heart muscle, and *sca-1* regulatory sequences to express protein in hematopoietic stem cells (Miles et al. *Development*, Vol. 124, (1997) pp. 537-547), or a retina-specific regulatory element of the interphotoreceptor retinoid-binding protein (Bobola et al. *J.Biol. Chem.* Vol 270, (1995) pp 1289-1294). Other transgenic mice may be formed in which a selected sequence from the hedgehog gene family may be placed under control of an enhancer and/or promoter of the sort described above. Furthermore, transgenic mice may be generated in which the hedgehog or hedgehog agonist or antagonist is expressed under the control of heterologous tissue specific promoters/enhancers such as described above. Other transgenic animals may be formed in which hedgehog regulatory sequences are used to drive expression of heterologous gene coding

sequences in specific embryonic or adult tissues eg *Ihh* regulatory sequences for driving the expression of *Shh* or *Dhh*.

Transgenic mouse models according to the above may be formed by the methodology described in Example 1. "Knock-in" mice may be made using the method of Hanks et al. *Science* vol 269 (1995)pp 679-682, to target hedgehog genes into selected sites in the genome under the control of endogenous sequences in embryonic stem (ES) cells. These modified ES cells may then be micro-injected into blastocysts to form chimeric animals. (Joyner 1995). These animals are heterozygous for the targeted gene and will misexpress the introduced sequences. In this manner, control of the level of gene expression and of the sites at which expression occurs may be achieved. An example of such a transgenic mouse would be one in which *Ihh* sequences are "knocked into" the endogenous *flk-1* locus to permit expression in HSC and endothelial cell precursors. The transgene ("knock-in" gene) can be modified as a fusion protein with for example LacZ or GFP, to permit convenient histochemical or immunological or molecular detection.

The use of transgenic animal technology can provide mouse model systems for applications including the following: identifying additional events in the normal processes of hematopoiesis and vascular growth in embryonic, fetal and adult mammals and events that give rise to blood diseases such as leukemias, and abnormal vascular growth and abnormal hematopoiesis. These events may be analyzed with regard to hedgehog compounds.

Therapeutic applications:

There are a number of therapeutic applications for compounds of the invention. Such uses are associated with the modulation of hematopoiesis and vascular growth and include methods that result in stimulation as well as those that result in inhibition of proliferation and/or differentiation of stem cells. Examples of compounds of the invention have been discussed above.

In embodiments of the invention, the method of stimulating hematopoiesis and vascular growth may utilize:

(a) therapeutic compounds such as hedgehog proteins including derivatives, analogs, and degradation products of naturally occurring proteins; agonists or antagonists of protein receptors as well as functional equivalents of the above listed compounds. The therapeutic compounds may be isolated from cultures of extra-embryonic tissues, manufactured by recombinant technology or prepared by synthetic chemistry;

(b) coding sequences for the above- listed therapeutic compounds, incorporated into vectors suited for gene therapy techniques; and

(c) mammalian cells that have been transformed with coding sequences of the above for cell transplantation.

Treatment of subjects with abnormal blood development can be achieved by administering, in an effective dose, for an effective time, a therapeutic agent that has been identified by one of the assays of the invention to the patient by any of the above methods. Alternatively, patients may be subjected to gene therapy by creating a plasmid or viral vector containing the coding sequence for the therapeutic agent using any of the techniques available in the art. For example, a protein, analogue, derivative, antagonist or receptor, of an identified protein (collectively called compounds) such as hedghog related compounds, may be introduced into a vector and the vector introduced into the appropriate target tissue where this tissue is located in an adult or in an embryo. The expression of the therapeutic agent may be regulated by a selected enhancer to ensure selective expression in the targeted tissue. For example, use of the cardiac actin enhancer to express the desired compound in the heart, the MCK enhancer to express the compound in skeletal muscle; *sca-1* regulatory sequences to express hedgehog compound in hematopoietic stem cells or a retina-specific regulatory element of the interphotoreceptor retinoid-binding protein to express the compound in the retina.

Subjects with abnormal blood development can be treated by administering the therapeutic agent by means of cell transplantation using genetically manipulated cell lines as delivery systems of the secreted agent. For example, autologous cells such as autologous fibroblasts or heterologous cells contained within an immune protective barrier, may be manipulated by standard techniques to secrete the selected protein such as hedgehog, or analogues, derivatives, antagonists or receptors of protein.

In an embodiment of the invention, methods are provided for stimulating hematopoiesis in a subject to treat abnormalities associated with deficiencies in hematopoietic cell lineages. Examples of targets for such treatments include *in vivo* or *in vitro* exposure of undifferentiated mesodermally derived cells to a compound of the invention. Examples of target cells include bone marrow stem cells, progenitor cells, and cord blood cells. These cells may be isolated from a subject and stored in a cell bank for subsequent use, or the cells may be freshly isolated and maintained *in vitro* in a culture medium. Exposure of such cells to the compound results in enhanced proliferation and/or differentiation of the cells, the stimulated cells being implanted in the same or different subject from which the cells were derived, by means of transplantation technology. Alternatively, undifferentiated mesodermally derived cells may be accessed in the embryo or adult *in vivo* by any of a number of routes including: oral, intradermal subcutaneous, transmucosal, intramuscular or intravenous routes.

The method of the invention may be used to treat subjects (embryo or adult) suffering from blood abnormalities. These may arise from genetic lesions, side effects of therapeutic treatments such as radiation and chemotherapy for cancer or from disease caused by infectious agents such as human immune deficiency virus and may be treated using a method and compounds that stimulate hematopoiesis. The consequences of such abnormalities if untreated are various forms of anemia (associated with abnormally low levels of erythrocytes). Examples of anemias include: aplastic anemia (idiopathic, constitutional forms, or secondary forms); myelodysplastic anemia; anemia in patients with metastatic or necrotizing carcinoma; Hodgkin's disease; malignant lymphoma; anemia of chronic liver disease; anemia of chronic renal disease (renal failure); anemia of endocrine disorders; red cell aplasia; idiopathic or associated with other disorders, anemia due to chronic inflammatory disease; and thrombocytopenia of many etiologies. In addition, stimulation of hematopoiesis is beneficial in the treatment of leukopenias (for example, leukemia and AIDS).

According to an embodiment of the invention, a method is further provided for treating abnormal blood vessel formation (hypervascularization) resulting from genetic diseases, chronic degenerative disease, aging, trauma, or infectious agents. Examples include diabetic chronic ulcers, burns, frost bite, ischemic events following stroke and transplantation. The compounds of

the invention may be used in the adult for induction of revascularization or formation of collateral vessels in ischemic myocardium or ischemic limbs, and in coronary artery bypasses and in promoting wound healing in general. For example, compounds of the invention may be used in treatment of duodenal ulcers by enhancing microvessel density and promoting more rapid healing. In addition, the method of the invention may be used to correct disorders of development in the embryo (as defined in above) caused by abnormalities in vascular growth.

According to an embodiment of the invention, methods are provided for inhibiting hematopoiesis in subjects suffering from excess production of erythrocytes for example polycythemia vera and erythroleukemia or other hematopoietic malignancies.

Similarly, methods are provided for inhibiting vascular growth in subjects suffering from excess vascularization or neovascularization as found in, for example, a variety of solid tumors such as breast cancer, hemangiomas in infancy, ocular neovascularization associated with diabetes, bleeding disorders of the female reproductive tract, and certain forms of arthritis.

All references cited above are incorporated by reference.

Examples

Example 1: Formation of transgenic mouse models to detect formation of primitive erythroid cells and hematopoiesis

Single erythroid cells formed during early embryogenesis can be identified by monitoring ϵ-globin expression. We developed novel ϵ-globin/LacZ vectors for transgene expression from which we obtained detailed histochemical data as well as data on the specificity of expression at the single cell level concerning hematopoiesis and vascular growth. Because the ϵ-globin/LacZ transgene is expressed only in primitive erythroid cells in mouse embryos (yolk sac and fetal liver), these mice serve as an ideal target for pharmacologic manipulation or examination of the effects of over-expressing or knocking out other genes that could affect embryonic hematopoiesis (which is primarily erythroid). For example, transgenic mice over-expressing a gene of interest may be crossed with one of the transgenic lines described above (homozygosed so that all progeny carry the LacZ reporter) and the effect on embryonic hematopoiesis measured by LacZ staining. Quantitative analysis of expression in tissue lysates were performed using the methods

described by Wassarman, et al., *Guide to Techniques in Mouse Development* (San Diego: Academic Press, Inc., 1993); Herbomel, et al., *Cell* 39 (1984), 653-62). Alternatively, mice carrying targeted mutations (null mutations or other more subtle mutations) may be crossed with our transgenic mice and the effects of the mutations on embryonic hematopoiesis assessed. This *in vivo* assay is therefore a powerful tool for evaluating the effects of gene products on embryonic hematopoiesis.

We designed the transgene in association with a reporter gene to provide a sensitive enzymic assay for determining expression. Consequently we inserted β-galactosidase (LacZ) reporter gene into a vector downstream of a number of regulatory elements associated with the transcription of the human embryonic β-like hemoglobin (ϵ-globin) gene (Fig.1). Examples of transgene constructs used to follow blood cell development are provided below. These constructs are illustrative of the method of the assay which need not be restricted solely to these constructs but may utilize other transgenes and other reporter genes in other vector constructs.

Description of transgenes:

Several different transgenic constructs were generated containing a "micro-LCR" (a truncated version of regulatory sequences located far upstream of the β-globin gene locus, ref.. Forrester et al., 1989) plus: the minimal ϵ-globin promoter alone (construct 1); the upstream regulatory region to -849 (construct 2); the upstream regulatory region to -2025 (construct 3); the minimal promoter driven by the combination ϵ-PRE II + V (construct 4) (Trepicchio, et al., *Mol. Cell. Biol.*, 13 (1993), 7457-7468). The prokaryotic β-galactosidase (LacZ) gene was inserted, along with a short oligonucleotide (SDK) containing a Kozak consensus sequence (Ravid et al.), between the minimal promoter and part of the second intron of the ϵ-globin gene, deleting E-globin sequences between +20 and +473. For each construct, 8-10 founders were obtained (14-21% transgenicity).

Generation of transgene constructs:

Construct 1: -179 lacZϵμLCR (MB70):

This was the "basic cassette" and was created using a series of cloning steps.

MHB135 was first generated by three-way ligation between a ClaI/EcoRI fragment containing the human ϵ-globin gene from -849 to +1746, a 2.5 kb EcoRI/HindIII fragment containing a modified μLCR (Trepicchio, William L., et al., *Molecular and Cellular Biology*, Vol. 13, No. 12, pp. 7457-7468, (1993)), and a ClaI/HindIII-digested derivative of SP73 (Promega) in which the XhoI site had been destroyed by XhoI digestion, reaction with Klenow DNA polymerase, and then blunt ligation. A KpnI linker was then inserted at the EcoRV site of MHB135 to create MHB135K. A BamHI/XhoI fragment from pUCϵx (Baron, et al., *Cell,* 46 (1986), 591-602.) containing the minimal ϵ-globin gene promoter from -179 to +20 was subcloned into BamHI/XhoI-digested SP73 (Promega), then excised by digestion with KpnI and XhoI and ligated into the backbone fragment of KpnI/XhoI-digested MHB135K to yield MB42.

MB42 was modified by insertion of a NotI linker at the HindIII site using standard methods (Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989); this step introduced a unique NotI site at the end of the μLCR, yielding MB60.

MB59 was next generated as follows. The ~3.1 kb XbaI/PstI LacZ fragment from pSDKlacZpA (REF) was subcloned into SP73 to give SP73lacZ. The 3' portion of the human ϵ-globin gene from +474 to +1746 was excised as a BamHI/EcoRI fragment from pUCϵx and blunt end-ligated into the blunted BamHI site of SP73lacZ to yield MB59. The KpnI site of MB59 was next destroyed by treatment with T4 DNA polymerase and relegation, yielding MB 69. Finally, the XhoI/EcoRI backbone of MB60 was ligated to a fragment containing LacZ and the ϵ-globin 3' sequences obtained by partial EcoRI and then XhoI digestion of MB69. This final product, MB70, was the basic construct, -179lacZϵμLCR.

Construct 2: -849lacZϵμLCR (MB73) :

The ϵ-globin upstream region from -849 to +20 was isolated as a BglII/XhoI fragment from MHB135 and subcloned into the backbone of BglII/XhoI-digested MB70 to yield -849lacZϵμLCR, denoted MB73.

Construct 3: -2kblacZϵμLCR (MB 92):

A fragment of 2kb containing the ϵ-globin upstream region from -2025 to +20 was excised from MB16-3 by digestion with KpnI and XhoI and was ligated into the backbone fragment of Kpn/XhoI-digested MB70-3 (-181lacZϵμLCR, see above) to generate -2kblacZϵμLCR (MB92).

MB16-3 was generated as follows. The 2kb ϵ-globin upstream region was isolated from pUCϵx (Baron and Maniatis, 1986) by digesting with EcoRI, blunting with Klenow DNA polymerase, and then digesting with XhoI. It was then ligated into KpnI/XhoI-digested MHB135K (see above).

Construct 4: ϵ-PRE(II+V)μLCR (~~MB72~~ (MB72).

This vector was generated by ligating the BglII/BamHI fragment from construct 6 of Fig. 4 from ref. (Trepicchio et al., 1993) into the BamHI site of the basic construct, MB70.

Generation of transgenic mice:

For microinjection into embryos, plasmid DNAs were digested with KpnI/NotI restriction enzymes (Fig. 1) and the eukaryotic portions purified using standard methods (Hogan et al., 1994). The embryos were microinjected at the single cell stage with the DNA samples and then implanted into foster mothers, using standard methods (**Hogan et al., 1994**). An outbred mouse strain (CD-1) was used for generation of transgenic mice and has served as a source of embryo donors, stud males, pseudopregnant females, vasectomized males, and mature females for breeding. Tail biopsies were genotyped by Southern blotting (using a number of different probes, again by standard methods) or PCR (see below). Southern blot analysis was also used to confirm that no rearrangements, duplications or deletions accompanied genomic integration of the transgene. Founders were bred to obtain transgenic males (heterozygous transgenic CD-1 males) which were mated with normal CD-1 females to produce embryos or adult animals for LacZ expression analysis (see Figure 2). Pregnant females were sacrificed at the times indicated in the figure (Noon of the day of vaginal plug observation was considered day 0.5 postcoitum (dpc)). Embryos were dissected, fixed and stained with XGal. For analysis of transgene

expression in staged mouse embryos, enzymatic β-gal activity was followed by staining of whole mount embryos using a standard method (Wassarman and DePamphilis, 1993).

PCR conditions for genotyping transgenic mice :

Genomic DNA was prepared from toe clips of 10 day old pups or from tail biopsies of 3 week old pups. Toe clips were added to 20 µl of DNA extraction buffer (50 mM Tris-HCl, pH 8, 20 mM NaCl, 1 mM EDTA, 1% SDS) containing protease K (1 mg/ml) and incubated for 1 hr. at 55°C with vortexing after the first 30 min. The samples were then diluted with 200 µl water, boiled for 10 min and microcentrifuged for 20 min. Genomic DNA was prepared from tail biopsies by a standard method (Hogan et al., 1994). PCR was carried out using 0.4 µl of genomic DNA (toe or tail) in a 50 µl reaction containing 1X Buffer G (PCR Optimizer kit, Invitrogen) and I.U. Ampli-Taq polymerase (Perkin-Elmer). Amplification was carried out for 32 cycles of denaturation at 94°C (1 minute), annealing at 55°C (1 minute), and extension at 72°C (1 minute) followed by a final 6 min extension at 72°C and resulted in a product of 408 bp. A portion (10-15 µl) of the reaction was analyzed on a 2% agarose gel in 1X Tris-borate-EDTA (Sambrook et al., 1989). The sequences of the amplification primers were:

5' Hε: 5'- ATG GAT CCA GCA CAC ATT A -3' (corresponds to -179 to -165 of Hε-globin gene) (SEQ ID NO:1)

3' LacZ: 5'- TCG CCA TTC AGG CTG CG -3' (corresponds to +154 to +170 of LacZ) (SEQ ID NO:2)

Results:

LacZ expression was detectable as early as 7.5 days post coitum (dpc) (i.e. about the time when blood islands are first seen in the yolk sac), in the expected "ring" pattern at the level of the exocoelom (Fig. 2A). By 8.5 dpc, staining of primitive erythroblasts within the vascular channels of the yolk sac was observed (Fig. 2B). Within the embryo proper, a small number of LacZ-staining primitive erythroblasts were observed (Fig. 2B(c)). By day 12.5, the time when mouse ε-globin gene expression peaks, LacZ-staining primitive erythroid cells were seen within yolk sac blood vessels (Fig. 2C). We have stained embryonic blood directly, to verify that these cells express LacZ.

Mice carrying MB 70, MB72 or MB73 expressed LacZ in primitive erythroid cells of the yolk sac and also in fetal liver at 16.5 dpc. Mice transgenic for MB 70 and MB 73 did not express LacZ in adult tissues. In contrast, in the absence of negative regulatory elements upstream of the promoter, MB72 transgenic mice also expressed LacZ in adult erythroid cells. MB 72 can therefore be used to study pharmacologic induction of anemias or polycythemia in adult animals. These mice may also be crossed with other transgenic or knockout mice to examine the effects on adult erythropoiesis of over-expressing or knocking out other genes. Examples of diseases which may be studied using these mice (after mating with other mice carrying appropriate mutations) include sickle cell anemia and the thalassemias (*e.g.*,Skow, L.C., et al., *Cell*, Vol. 34, pp. 1043-1052, (1983); Ciavatta, D.J., et al., *Proc. Natl. Acad. Sci. USA*, Vol. 92, pp. 9259-9263, (1995)).

With respect to yolk sac expression, primitive erythroid cells were LacZ-positive in MB 92 mice, similar to that detected for mice carrying any of the other three constructs.

Example 2: Demonstration that the primitive embryonic mesoderm by itself cannot give rise to hematopoiesis and vascular growth; Embryo explant cultures are used to identify agents that stimulate hematopoiesis and vascular growth in these cultures

Mice transgenic for a LacZ reporter linked to one of several human embryonic β-globin upstream regulatory sequences (Fig. 1) have been bred to homozygosity. These animals serve as a source of marked embryos in which the transgene is expressed only in primitive erythroid cells (Fig. 2).

(A) THE VISCERAL ENDODERM IS REQUIRED FOR PRIMITIVE HEMATOPOIESIS.

The Embryo Explant Culture: Embryos from the transgenic mice of Example 1 were isolated around the onset of gastrulation at 6.25-6.5dpc prior to the formation of hematopoietic mesoderm and were maintained individually in the chambers of an 8-well slide (Costar) or the wells of a 24-well plate (Costar) or in individual wells of Terasaki plates (Nunc) or in the wells of a 4 well plate for 48-72 hr. The embryos were then fixed and stained with XGal using a standard protocol (Wasserman, P. M. and Melvin L. DePamphilis, eds., *Guide to Techniques in Mouse Development,* Vol. 225, pp. 461-463. 1993) to monitor the generation of primitive

erythroblasts. Whole embryos were cultured either in serum-containing medium or in chemically-defined medium (CDM) for LacZ-positive blood islands. CDM was similar to that used by (Johansson and Wiles, 1995) except that penicillin (1,000 U/ml), streptomycin (1,000 µg/ml), and Hepes pH 7.4 (20 mM) were added.

Separation of Visceral Endoderm from Epiblast: Pre-streak to early-streak embryos were enzymatically separated (Farrington, S.. M., et al., *Mechanisms of Development*, Vol. 62, pp. 197-211, (1997)) into ectodermal (epiblast) and visceral endoderm components using trypsin/pancreatin (15 sec to 2 min) using a standard technique (Hogan et al., 1994). Tissue cross-contamination during this procedure was found to be negligible. (Farrington et al. 1997). Epiblasts or whole embryos were cultured individually. Figure 2a depicts the epiblast which is a descendant of the inner cell mass of the blastocyst, from which ES cells are derived.

The visceral endoderm is required for primitive hematopoiesis in the mesoderm.

(a) Whereas LacZ-positive blood islands were easily detected in whole embryo cultures, little or no LacZ staining was observed in the epiblast cultures, either in chemically defined medium (CDM) or in Dulbecco's Modified Eagle's Medium (GIBCO-BRL) containing 30% heat-inactivated (56°C, 30 min) fetal bovine calf serum (HyClone). These results demonstrate that the mesoderm cannot on its own give rise to embryonic hematopoiesis but requires contact with or signals released from visceral endoderm. In contrast, epiblasts taken from later (6.75 to 7.5 dpc) embryos do form blood islands after 48 hours in culture, presumably because mesodermal cells present at this stage will already have received signals from the visceral endoderm.

(b) Recombination experiments: Epiblasts were recombined with visceral endoderm in collagen gels (rat tail collagen type I, Collaborative Biomedical Products). Collagen was prepared according to the instructions of the manufacturer. A 10 µl drop of collagen was allowed to solidify on the plastic surface; the tissues were then juxtaposed in a small depression created using watchmaker's forceps and then covered with 1 µl of collagen to hold them in place. Alternatively, tissues were gently expelled into a 5 µl drop of collagen and juxtaposed to allow physical contact; the collagen was then permitted to solidify. After 10 min, explant culture medium (DME supplemented with 30% FBS (heat-inactivated @ 56°C, 30 min), 2 mM

glutamine, 10 mM Hepes pH 7.4, 68 μM α-methyl thioglycerol, penicillin (1,000 U/ml) and streptomycin (1,000 μg/ml) was added to the well (0.5 ml for 24 well dishes, less for smaller wells). Embryo fragments were manipulated using a drawn-out Pasteur pipet. All cultures were maintained at 37°C and 5% CO_2. Medium was changed after one day. RNA was harvested after 3 days using the small scale method of (Chomezynski, P., et al., *Anal. Biochem*, Vol 162, pp. 156-159, (1987)) and analyzed for embryonic globin gene expression by RT-PCR (Farrington et al., 1997). These experiments demonstrated activation of globin gene expression when epiblasts were recombined with visceral endoderm and therefore established a requirement for visceral endoderm in induction of hematopoiesis during gastrulation (Figs. 7, 8-1).

(c) Activation of primitive erythropoiesis by a diffusible factor in visceral endoderm cells: END-2 (Mummery et al., *Dev. Biol.*, 109 (1985), 402-410) - a visceral endoderm derived cell line, was grown to confluence in DME containing 15% fetal bovine serum (FBS) Cells were trypsinized , resuspended in 5mls DME containing 30% FBS (DME-30) and gamma irradiated (6000 rad) using ^{137}Cs source. Cells were pipetted to break up the clumps and were then added to an additional 15mlDME-30 in a 10cm dish. The cells were allowed to condition the medium for 3 days at 37°C (5%) CO_2. The medium was harvested , residual cells removed by centrifugation at 10 min at 1500rpm and the supernatant was then sterilized using a 0.2mm filter. The resulting conditioned medium (CM) was stored in aliquots at -80 °C.

Epiblasts were incubated with (+) or without (-) CM. Most of the epiblasts cultured without CM failed to activate a marker of primitive erythropoiesis (the ϵ-globin gene), while most of the epiblasts cultured in the presence of CM did activate the gene. These results suggest that cell-cell contact is not essential for the stimulation of hematopoiesis by visceral endoderm, but that the effect is mediated by one or more diffusible factors. The asterisk in Fig. 10 indicates an artifactual amplification product.

The results show that for whole untreated embryos, 6/6 produced globin. In contrast, of 8 untreated epiblasts, only 1 showed any detectable expression. When conditioned medium was added, 8/10 epiblast cultures expressed globin.

Determination of the time at which hematopoiesis first occurs during mouse development

In situ hybridization and histology:

Whole-mount *in situ* hybridization was carried out as in Wilkinson and Nieto, 1993, using BM Purple (Boehringer Mannheim) as the substrate for alkaline phosphatase detection. The probe used is mouse ϵ-globin probe. The digoxigenin-labeled riboprobe was prepared by T7 polymerase transcription from an EcoR1-linearized DNA template termed SP73 mϵRB, using a standard method (Wilkinson and Green, *Postimplantation Mouse Embryos: A Practical Approach.* Ed. A. Copp. Oxford: IRL Press, 1990). SP73 mϵRB was generated by ligation of the EcoRI-BamHI fragment of the mouse ϵ-globin gene (Baron and Maniatis, 1986) from +187 to +439. This fragment contains a small region of the first intron and most of the second exon of this gene. The results of *in situ* hybridization on whole embryos to determine time at which hematopoiesis could be detected in wild type mice is shown in Fig. 5.

Multiplex RT-PCR protocol was used to measure induction of hematopoiesis because it is a more sensitive and quantitative assay for induction of hematopoiesis than XGal staining. It is also more versatile than XGal staining because it allows analysis of the expression of a variety of genes in a tissue. The starting material for this technique is RNA. Oligonucleotide primers were prepared. Examples of primers are provided in Table 1. Total RNA was prepared by guanidinium-acid-phenol extraction (Chomczynski, et al. (1987)) from the tissues of single embryos (6.25 to 6.5 dpc samples).

Total RNA was reverse transcribed with AMV reverse transcriptase (Life Sciences, Inc.) by standard methods, using oligo(dT) primer (Sambrook et al., 1989). Multiplex PCR was performed in a 15 μl reaction containing 5 pmol of β-actin primers (as an internal standard), 10-45 pmol of test gene primers and a trace amount of [α-^{32}P]-dCTP to enable detection of amplification products by autoradiography following polyacrylamide gel electrophoresis. The primers used for PCR are described in Table 1. Amounts of input cDNA were normalized for β-actin expression. The cycle number and amounts of primer and template cDNA which yielded non-saturating amplification were determined empirically in each case.

Embryonic β-like globin (ϵ) gene expression was not detected in 6.5 dpc epiblasts or whole embryos isolated at 6.25 to 6.5 dpc. After 72 hr in culture, the ϵ-globin gene was activated in whole embryos but in isolated epiblasts little or no ϵ-globin transcription could be

detected (Figs. 7, 8). This demonstrated that embryonic hematopoiesis was not mesoderm-autonomous and that induction of embryonic globin gene expression occurred in the presence of visceral endoderm. This effect was consistent with a requirement for visceral endoderm for induction of embryonic globin gene expression. (The observed effect could be readily distinguished from the predicted effect of random events resulting from variations in embryo development in a litter in which isolated epiblasts at a more advanced stage of development at the time the embryo was harvested may provide low levels of globin gene expression).

(B) BLOOD FORMATION IN THE ANTERIOR PORTION OF AN EPIBLAST OBTAINED FROM A LATE-STAGE GASTRULATING EMBRYO.

Individual late stage gastrulating embryos (around 6.75dpc) were harvested and the surrounding visceral endoderm was removed. At this time, the epiblast has already received visceral endoderm signals and has developed the capacity to form blood. However, this capacity appears to be localized at this time to the posterior region of the epiblast. We have shown here that the anterior region retains its dependence on the extraembryonic visceral endoderm. Visceral endoderm was enzymatically removed as described by Farrington et al. (1997) and the extraembryonic ectoderm was dissected away. The epiblast (embryonic ectoderm) was transected into anterior and posterior sections and incubated separately for 3 days. The posterior section was identified on the basis of morphological landmarks such as the primitive streak (posterior epiblast), as described by Downs et al. *Development,* vol. 118 (1993) 1255-1266 (Figure 15). The results are shown in Figure 5 and 16-1, 16-2, 16-3 and 16-4 and discussed below.

(i) During late stage gastrulation, the posterior but not anterior portion of the embryo is capable of forming blood in the absence of visceral endoderm

Using RT-PCR as described above, little or no ϵ-globin expression was observed in the anterior portion of the embryo nor could blood development be observed histologically. In contrast, the posterior section formed blood even in the absence of the visceral endoderm, at levels comparable to that of an intact embryo (shown as control). Controls included (i) a PCR reaction carried out in the absence of cDNA template; (-c-DNA), (ii) RNA incubations carried

out using a reverse transcription cocktail without reverse transcriptase (ant (-RT), post (-RT), Farrington et al, 1997). In this experiment, actin was amplified for 18 cycles and globin was amplified for 23 cycles.

(ii) Signals from visceral endoderm can restore ability of anterior portion of late gastrulation stage embryos to form blood.

(a) Four anterior and four posterior epiblast portions of late primitive streak stage embryos were cultured individually in the absence of visceral endoderm. As observed in the experiment of Fig. 2A, the posterior but not anterior portions of these late gastrulation stage embryos were able to form blood. In contrast, when anterior epiblast portions were cultured in collagen drops with visceral endoderm, blood formation was reconstituted in 2 of 4 samples (denoted "recombs" in figure). In this experiment, actin was amplified for 21 cycles and globin was amplified for 26 cycles.

(b) We determined that blood formation by mesoderm requires signals from visceral endoderm as follows: transgenic (Tg) embryonic ectoderms (epiblasts) were stripped of their visceral endoderm (VE) and recombined with non-transgenic VE in droplets of collagen. In these experiments, the only possible source of hematopoietic cells would be the transgenic epiblast but not the non-Tg VE. Tissues were cultured for 3-4 days and then stained with XGAL to identify areas of lacZ expression. These experiments demonstrated that blood formation was reconstituted in the presence of VE tissue. Furthermore, the localization of blood cells to the area immediately around the VE tissue suggested either that direct cell-cell contact is required or that short-range signaling by diffusible molecules is involved (Figure 8-1).

(c) We determined that the visceral endoderm can reprogram the anterior embryonic ectoderm of the epiblast to express both hematopoietic and vascular endothelial markers as follows: The anterior epiblast was recombined with visceral endoderm, to obtain activation of both hematopoietic markers (ϵ-globin, GATA-1 , CD-34) shown in Fig 16-2 and endothelial (PECAM-1, flk-1, and Vezf-1,) markers shown in Fig. 16-3. These markers were strongly expressed in cultured posterior epiblasts isolated at mid- to late gastrulation (Figure 16-2, posterior lanes 6-10, and Figure 16-3, posterior lanes 6-10) and in cultured whole embryos (emb+cx). Little or no expression of these markers was detected in visceral endoderm alone

(Figure 16-2, anterior lanes 6-10; Figure 16-4, anterior, lanes 6-10) or in uncultured whole embryos (Figure 16-2, emb (-cx); Figure 16-3, emb (-cx)) from the same stage of development. These experiments showed that both hematopoietic and vascular tissue were induced by visceral endoderm signals and that the signals were instructive. As a specificity control, cardiac myosin which was expected to be expressed in cardiac tissue (and therefore only at a later developmental stage, around 7-8 dpc) (Lyons et al. 1990) was not detected in anterior or posterior epiblasts or in recombinants during the first 3 days in culture (Figure 16-2, lanes 3-17) but was detected in a 10.5dpc embryonic control (Figure 16-2, 16-3). Vezf-1 (5') and (3') primers yielded a product of approximately 700 bp. Vezf-1 is a zinc finger protein homologous to a human protein termed db-1 and is expressed in the developing vasculature. It was shown to occur predominantly in the posterior epiblast and recombinant epiblasts but not in the anterior epiblasts (Fig 16-3)

PCR primers used to identify expression of markers

Primer sequences:

GATA-1 (5') :	5'-CAGCACTAGGCCTACTACAG-3'	PCR product is 237bp; 32 cycles (SEQ ID NO:3)
GATA-1 (3')	5'-TCAAGGTGTCCAAGAACGTG-3'	(SEQ ID NO:4)
Bra(5')	5'-TGCTGCCTGTGAGTCATAAC-3'	PCR product is 741bp; 34 cycles (SEQ ID NO:5)
Bra (3')	5'-CTACTCTAAGGCAACAAGCC-3'	(SEQ ID NO:6)
Otx-2 (5')	5'-AGGAGCTGAGTCGCCACCTC-3'	PCR product is 312bp; 34 cycles (SEQ ID NO:7)
Otx-2 (3')	5'-GTAGCCCACGGAGGGATGCA-3'	(SEQ ID NO:8)
CD34 (5')	5'-GTTACCTCTGGGATCCCTTC-3'	PCR product is 612bp; 32 cycles (SEQ ID NO:9)
CD34 (3')	5'-GAGGTGACCAATGCAATAAG-3'	(SEQ ID NO:10)
PECAM-1 (5')	5'-TGCGATGGTGTATAACGTCA-3'	PCR product is 384bp; 32 cycles (SEQ ID NO:11)
PECAM-1 (3')	5'-GCTTGGCAGCGAAACACTAA-3'	(SEQ ID NO:12)
Flk-1 (5')	5'-CCATACCGCCTCTGTGACTT-3'	PCR product is 507bp; 32 cycles (SEQ ID NO:13)
Flk-1 (3')	5'-ACACGATGCCATGCTGGTCA-3'	(SEQ ID NO:14)
c-myosin(5')	5'-CTCGCAGAACAGCAGCCTAA-3'	PCR product is 679bp; 32 cycles (SEQ ID NO:15)
c-myosin(3')	5"-AGGGTCTGCTGGAGAGGTTA-3'	(SEQ ID NO:16)

(C) BLASTOCYSTS ISOLATED AT ABOUT 3.25-3.5DPC PROVIDE A MODEL SYSTEM FOR SCREENING COMPOUNDS THAT CAN STIMULATE HEMATOPOIESIS AND VASCULAR GROWTH OF UNDIFFERENTIATED MESODERMAL CELLS

Blastocyst cultures were prepared and used to analyze the effects of compounds on the stimulation of undifferentiated mesodermal derived cells to undergo hematopoiesis and vasculogenesis. The blastocyst culture system described here is suited for following the development of embryonic structures *in vitro*, such as the yolk sac, that normally form post implantation *in vivo*. The effects of exogenously added growth factors or signaling molecules on development are analyzed here under defined conditions. Blastocysts may be obtained from wild type mice, transgenic mice or knock-out mice. Embryonic hematopoiesis in knockout mice was studied using null mutant blastocysts, obtained by crossing heterozygous animals. These null mutant blastocysts are preferred over null mutant embryonic stem (ES) cells used *in vitro* differentiation assays (Keller, *Current Opin. Cell Biol.*, 7 (1995) 862-69) because of their greater ease of isolation.

The blastocyst assay relies on the recovery of embryos from mice at a time prior to implantation of the embryo into the uterus of the mother at about 4.5 dpc. Here, blastocysts were obtained from (a) ϵ-globin/LacZ transgenic mice prepared according to Example 1 and analyzed by Lac Z staining; and (b) non-transgenic mice or knockout mice where individual blastocysts were analyzed at the molecular level for expression of multiple genes by RT-PCR.

Blastocysts were harvested at 3.25 to 3.5 dpc as described by Robertson, *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach.* Oxford: IRL Press, 1993. We have successfully cultured blastocysts from several different mouse strains. In this example, CD-1 mice were used. The culture method was based on the method of Chen and Hsu (Chen, et al., *Exp. Hemat*, 7 (1979) 231-44.). However, superovulated females were not used. After harvesting, the blastocysts were washed free of contaminating maternal blood cells by two to three sequential transfers in drop cultures under mineral oil (Robertson, pp 471-478, ed IRL Press 1987) and transferred into untreated 35 mm plastic tissue culture dishes or into the wells of a 24 well dish. The blastocysts adhered to the plastic, reorganized and grew. Blastocysts were

cultured individually (in wells of 24 well dishes) or in groups (35 mm plates or 24 well dishes) of up to 20, in CMRL-10 medium for the first 48 hr and then in CMRL-20 for up to 10 days at 37°C and 5% CO_2. CMRL-10 was CMRL1066 medium (GIBCO-BRL) containing 10% heat-inactivated fetal bovine serum, penicillin (2,000 U/ml), streptomycin (2,000 µg/ml), 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids (GIBCO-BRL), and 10^{-4}M β-mercaptoethanol. Sac-like structures could first be seen around 7 days in culture; by 9-10 days they had enlarged to the point where they were easily visible with the naked eye (0.5-2 mm in diameter). These sac-like structures (here termed "blastosacs") closely resembled early murine yolk sacs.

Transgenic blastosacs were stained *in situ* for LacZ expression using standard methods (Wassarman and DePamphilis, 1993). For analysis by RT-PCR, individual blastosacs were transferred into eppendorf tubes using a P200 pipetman and were microfuged for 10 min at 4°C. Medium was aspirated and RNA was isolated from tissue pellets (Chomczynski et al. 1987). A portion (5 to 8 µl out of 25 µl) of the RNA was used for synthesis of cDNA (Farrington et al., 1997 incorporated by reference). 0.5 to 2 µl of RNA were amplified by PCR in 50 µl as described (Farrington et al., 1997). In Figs. 4, samples underwent 35 cycles of amplification and 10 µl of the reaction mixture was then analyzed by electrophoresis through a 2% agarose gel containing ethidium bromide.

Actin and mouse GATA-1 PCR primers have been described previously (Baron et al., *Molecular and Cellular Biology*, vol 14, (1994) pp. 3108-3114). All other primers were used at an annealing temperature of 55°C. Primer sizes were: mouse ϵ-globin, 487 bp; mouse NF-E2, 257 bp; mouse EKLF, 129 bp; PTH/PTHrP receptor, 279 bp; PTHrP, 421 bp. Primer sequences were:

Mϵ forward: 5'- GGA AAA AAC CCT CAT CAA TG -3' (SEQ ID NO:17)

Mϵ reverse:
5'- ATT CAT GTG CAG AGA GGA GGC ATA -3' (SEQ ID NO:18)

mNF-E2 forward: 5'- cga CTA GTT CGG GAC ATC CG -3' (lower case letters indicate SpeI site) (SEQ ID NO:19)

mNF-E2 reverse: 5'- atg gta ccG TAC ATA TTC CTC TGG TG -3' (lower case, KpnI site) (SEQ ID NO:20)

(SEQ ID NO: 21)

EKLF forward: 5'- cga cta GTG GCG GTC TGA GGA GAC -3' (lower case, SpeI site)

(SEQ ID NO: 22)

EKLF reverse: 5'- atg gta ccA CGC ACA GGT CAC GT -3' (lower case, KpnI site)

Hemoglobinized tissue was identified in these "blastosacs" following staining with benzidine (Fig. 3b). Benzidine staining corresponds to the presence of hemoglobin in erythroid cells. This was confirmed by analyzing embryos from transgenic mice. In these mice, LacZ expression was observed only in primitive erythroblasts but not in other embryonic cell types. Transgenic blastocysts were cultured for 9 days and then stained with X-Gal. As can be seen from Fig. 3C, erythroid cells produced in the developing blastosacs are easily revealed by their blue color after staining. Both wild type blastosacs and those derived from transgenic marker lines appear to contain vascular channels and resemble early embryonic yolk sacs (Fig. 3D) rather than later yolk sacs with well developed vasculature (compare with Fig. 2). The culture method described above provided blastosacs with an efficiency of between 40-80%.

RT-PCR was used to identify the temporal pattern of expression of mesodermal and endodermal markers in developing blastosacs and the effects of different growth factors and extracellular matrix components on the formation of different cell types. As shown in Fig. 4A, embryonic globin is produced only when yolk sac-like structures form, but not if the blastocysts do not progress in their development beyond an amorphous mound of trophectoderm cells.

Null mutant embryos were analyzed to determine the effects on hematopoiesis and vascular growth of mutations introduced into the mouse germline by gene targeting. Blastocyst cultures were harvested at 3.5dpc from heterozygous Bmp-4 knockout mice (Winnier, G., et al., *Genes & Development*, Vol. 9, pp. 2105-2116, (1995)) that were crossed to give rise to homozygous null mutant offspring. The blastocysts were incubated in culture for varying periods of time (for example 9 days), after which time, individual blastosacs were removed from the culture plate for RT-PCR analysis, leaving behind the trophectodermal tissue. This tissue was used for genotyping so as to establish that the transgenic mice from which the blastocysts are derived, were homozygous. Whole-mount *in situ* hybridization and immunohistochemistry was also used to identify presumptive mutants for mutations resulting in a severe deficiency in erythroid cells or endothelium.

Null mutant "embryoid bodies" Embryoid bodies are structures derived from ES cells that form blood islands under appropriate culture conditions (Keller (1995)). We have developed an assay system using embryoid bodies to show that null mutant embryoid bodies such as *Bmp-4,* form little or no blood, and that this defect can be rescued by addition of exogenous, recombinant protein (BMP-4). The ES cells used in this experiment were derived from the TL-1 subline. However, the assay conditions described below are effective for a number of other ES cell sublines. They are also effective whether the cells have been selected for feeder independence or are maintained on feeder cells (Joyner (1195) Gene Targeting: A Practical Approach (New York : IRL Press). with mutations in selected genes were rescued by addition of a compound that is functionally equivalent to the gene product expressed by the non- mutated gene.

ES cells carrying a null mutation in both alleles of the *Bmp-4* gene were formed using standard techniques (Joyner (1995) Gene Targeting: A Practical Approach. (New York:IRL Press), Keller, Current Opin. Cell Biol. 7, (1995)862-869; Orkin Current Opin. Cell Biol. 7 (1995) 870-877, Mortensen, Molec. Cell Biol. 12 (1995) 2391-2395). These cells were plated at about 2.5 x 10^5 cells on a 6 cm bacterial dish containing 5 ml of IMDM/15% serum (either plasma derived serum or a 1:1 mixture of PDS and fetal bovine serum). The addition of exogenous growth factors such as erythropoietin or IL-3 was not found to be necessary here, despite a large literature that claims that different cocktails of growth factors are essential (e.g. Keller 1995,). After 24 hr, ES cells had formed aggregates and these were gently resuspended in the same medium and plated in 0.8% methylcellulose in IMDM containing 10% serum with or without BMP-4 (recombinant human, from Genetics Institute; 2 ng/ml). Figure 17 (A) and (C) show wild type (parental) TL-I cells at low (A) and high (C) magnification. 87% of embryoid bodies from wild type ES cells became hemoglobinized after 10 days (see table at top of figure). In contrast, only 4% of the embryoid bodies from null mutant ES cells (B) became hemoglobinized. When BMP-4 was added to the cultures (D), the number of embryoid bodies to increased to about 59%. These results were confirmed using semi-quantitative RT-PCR assay for embryonic B-globin described above. These results show that inhibition of hematopoiesis can occur as a result of a mutation in the *Bmp-4* gene and this deficiency can be reversed by the addition of exogenous BMP-4 protein.

Example 3: Compounds that are functionally equivalent to a gene product expressed in an embryo's extraembryonic tissue (exemplified by hedgehog protein) stimulate hematopoiesis and vascular growth of undifferentiated mesodermal cells (exemplified by epiblast mesoderm)

(a) A hedgehog protein, typified by Sonic hedgehog, was demonstrated to stimulate hematopoiesis in the epiblast mesoderm using the method of Example 2(A) (Fig. 9). Bacterially expressed amino-terminal SHH protein (Bumcrot et al., 1995) in 20 mM Tris-HCl pH 7.6, 250 mM NaCl, 5% glycerol, and 1 mM DTT was diluted to 1 µg/µl in 10 mg/ml bovine serum albumin (Stem Cells Technology). SHH protein was added at various concentrations (0.25 µg/ml to 5 µg/ml;) to explant culture medium. Medium was changed after one day and RNA (Chomczynski and Sacchi, 1987) was isolated for RT-PCR analysis. Figure 9 shows that SHH protein can substitute for visceral endoderm in a dose-dependent manner.

(b) Compounds that are functionally equivalent to a gene product expressed in an embryo's extraembryonic tissue (exemplified by hedgehog protein) stimulate hematopoiesis and vascular growth of undifferentiated mesodermal cells (exemplified by adult bone marrow cells).

To determine whether recombinant hedgehog proteins influence the development or differentiation of adult hematopoietic stem or progenitor cells, we carried out *in vitro* clonal assays. Mononuclear cells isolated from murine bone marrow were plated in methyl cellulose as follows:

Bone marrow hematopoietic progenitor assays:

Bone marrow was flushed from femur and tibias of from 2 to 3 female ICR mice, aged 5-6 weeks, by a standard method (Lord, in *Haemopoiesis: A Practical Approach*, pp 1-53, ed. Testa and Molineux, 1993 pub. Oxford University Press) and transferred to 5 ml of alpha medium (GIBCO-BRL) containing 2% fetal calf serum (hyClone). Mononuclear cells from pooled samples were isolated by centrifugation on a cushion of Ficoll (Accurate Chemical Co.) (Testa and Molineux, 1993) and cell numbers determined using a Coulter Counter. Cells were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) at 3 x 10^5/ml and plated in a mixture of methyl cellulose (Fisher Scientific, 1.2%) in IMDM containing fetal calf serum (10%), deionized bovine serum albumin (cell culture grade BSA, 1%), 2-mercaptoethanol (1x10M) and the indicated growth factors and recombinant hedgehog proteins. Recombinant

human erythropoietin (Epo) was obtained from Amgen and used at 40 U/ml. Recombinant interleukin-3 (IL-3) and granulocyte/macrophage-colony stimulating factor (GM-CSF) were used at 50 U/ml each. Portions (0.3 ml) of the methyl cellulose-mononuclear cell mixture were plated into 3 wells of each of two 4 well dishes (Nunc) for each growth condition tested. The fourth well of each dish contained dH_2O to maintain humidity. Cultures were incubated in 5% CO_2 at 37°C for approximately 2 weeks and colony numbers were scored on the days indicted. Colonies were scored as CFU-E, BFU-E, myeloid or mixed. Where included in the cultures, recombinant hedgehog proteins were added at concentrations between 1 and 5 μg/ml. Buffer alone (5 mM sodium phosphate pH 5.5 150mM NaCl, 0.5 mM DTT) was added to some cultures as a negative control. For each culture condition, data were compile from counts of the 3 wells from each of two plates (6 wells total) +/- standard deviations.

The mononuclear cells isolated from bone marrow were plated in methylcellulose containing hematopoietic growth factors alone (erythropoietin only; or GM-CSF + IL-3; or the combination Epo + GM-CSF + IL-3) or supplemented with one of histidine-tagged amino-terminal peptide of SHH (SHH-HIS), amino-terminal peptide of SHH (SHH-N), or histidine-tagged amino-terminal peptide of IHH. Cultures containing growth factors alone or growth factors plus buffer were used as negative controls.

In three independent experiments, colony numbers of all types (erythroid: CFU-E, BFU-E; myeloid: CFU-GM) were increased by -1.5 to more than 4-fold, in a dose-dependent manner (recombinant hedgehog protein added at 1, 2.5, 5μg/ml, X ug). The observation that hedgehog proteins are apparently not selective for erythroid versus myeloid lineage is consistent with the hypothesis that they stimulate stem or early progenitor cell development. All three recombinant hedgehog proteins stimulated colony formation. From these data we conclude that both SHH and IHH enhance proliferation, differentiation and/or survival of hematopoietic stem/progenitor cells in vitro, even in the presence of one or more hematopoietic growth factors.

TABLE 1

Stimulation of bone marrow progenitor cells by recombinant HH proteins[1]

Addition	Concentration (μg/ml)	CFU-E (day 2)	fold increase[2]	BFU-E (day 7)	fold increase	CFU-GM (day 11)	fold increase[2]
none		94.6±8.2		18.7±3.9		20.9±0.9	
buffer		84.7±2.4		14.7±5.0		27.1±0.5	
IHH-HIS	1.0	134±29.2	1.49	19.4±3.7	1.16	27.1±6.0	1.1
	2.5	150±31.5	1.67	15.8±2.6	0.95	29.3±6.5	1.2
	5.0	156±17.0	1.74	15.0±3.6	0.90	42.5±2.3	1.8
SHH-N	1.0	138±30.0	1.54	14.3±4.6	0.86	31.9±5.4	1.3
	2.5	143±31.5	1.59	20.2±0.5	1.23	34.5±9.9	1.4
	5.0	154±25.2	1.72	21.3±1.0	1.28	31.9±4.1	1.3
SHH-HIS	1.0	197±19.2	2.20	24.6±1.0	1.47	30.8±4.1	1.3
	2.5	153±24.7	1.71	16.9±1.9	1.01	32.6±6.1	1.4
	5.0	146±13.9	1.63	29.7±3.2	1.78	43.4±8.9	2.0

[1] All cultures contained EPO (2U/ml), IL-3 (50 U/ml) and GM-CSF (2ng/ml) plus the indicated addition (none, buffer or HH protein).

2 Fold increase calculated based on average of the two control values (no addition or buffer only).

TABLE 2

Stimulation of bone marrow progenitor cells by recombinant HH proteins[1]

Addition	concentration (μg/ml)	CFU-E (day 4)	fold increase[2]	BFU-E (day 8)	fold increase[2]	CFU-GM (day 8)	fold increase[2]
none		31		14		20	
buffer pH 8.0[3]		44		16		21	
buffer pH 5.5[3]		34		29		27	
IHH-HIS	5	63	1.75	25	1.25	25	1.09
	10	88	2.44	29	1.45	45	1.96
	25	130	3.61	31	1.55	43	1.87
SHH-N	5	71	1.97	47	2.35	28	1.22
	10	76	2.11	42	2.10	27	1.17
	25	136	3.78	46	2.30	28	1.22
SHH-HIS	5	112	3.11	27	1.35	38	1.65
	10	101	2.81	24	1.20	41	1.78
	25	111	3.08	29	1.45	45	1.96

[1] Erythroid colonies (CFU-E and BFU-E) were counted for cultures containing Epo (2U/ml) plus the indicated addition (none, buffer or HH protein). Myeloid colonies (CFU-GM) were counted for cultures containing IL-3 (50 U/ml) and GM-CSF (2 ng/ml) plus the indicated addition.
[2] Fold increase was calculated based on average of the three control values (no addition or buffer only).
[3] HIS-tagged proteins were stored in buffer pH 8.0; untagged SHH was stored in buffer pH 5.5.

Other approaches to measuring the effect of compounds that are functionally equivalent to a gene product expressed in an embryo's extraembryonic tissue on undifferentiated mesodermal cells:

An *in vivo* CFU-S spleen colony assay for multipotential and marrow repopulating cells was performed by injecting a source of hematopoietic stem/progenitor cells into mice. Macroscopic colonies formed in the spleen after 8-10 days reflected the presence of stem/progenitor cells (Testa and Molineux, 1993). As is the case for the *in vitro* progenitor assay described above, the maturity of the colony was reflected in the time taken for the colony to

develop: early appearing colonies represented more mature progenitors while later-appearing colonies represented more primitive progenitors.

In a separate experiment, stem/progenitor cell populations from murine and human hematopoietic tissue are enriched by flow cytometry (florescence-activated cell sorting, FACS) or magnetic immunoselection (Testa and Molineux, 1993) and their development enhanced in the presence of hedgehog protein. These resulting populations are examined using in vivo assays include the CFU-S assay (spleen colony-forming unit) and long-term bone marrow cultures . A typical bone marrow culture includes a competitive repopulation assays and serial bone marrow transplantation studies (Morrison, et al., 1995a; Morrison et al., 1995b).

Example 4: Inhibition of primitive erythropoiesis in cultured whole embryos using a SHH blocking antibody.

Whole embryos from two litters of mice were isolated at about 6.5dpc and cultured individually in the absence of exogenous IgG (none) or in the presence of purified IgG (46 μg/ml) (Ericson et al., *Cell* 87 (1996), 661-73). Expression of embryonic ϵ-globin was assayed by the semi-quantitative RT-PCR method. The results are shown in Figure 11. The asterisk indicates an artifactual amplified product. As predicted from Experiment 3, ϵ-globin expression was substantially reduced in the presence of the SHH blocking antibody.

Example 5: Cell Receptors *patched* and *Gli* are targets for stimulation of hematopoiesis and vascular growth.

Using the methods of Example 2(b), we showed that gene expression of *patched* and *Gli* was substantially exclusive in the yolk sac mesoderm. (Fig. 6) The enriched expression of *Gli* and *patched* in yolk sac mesoderm points to mesoderm as target of hedgehog signalling.: Yolk sacs from 10.5 and 12.5 dpc embryos were separated into endoderm (e) and mesoderm (m) fractions and RNA was prepared as described by Farrington et al (1997). RT-PCR analyses were carried out as described in Example 3 above using the following primers:

(SEQ ID NO 25)

Gli-1 5': 5'- CAG GGA AGA GAG CAG ACT GA -3' (+465 to +484 of sequence)

(SEQ ID NO 24)

Gli-1 3': 5'- AGC TGA TGC AGC TGA TCC AG -3' (+697 to +716 of sequence)

(SEQ ID NO 25)

ptc 5': 5'- CTG CTG CTA TCC ATC AGC GT -3' (+3040 to +3059 of sequence)

(SEQ ID NO 26)

ptc 3': 5'- AAG AAG GAT AAG AGG ACA GG -3' (+3491 to +3472 of sequence)

An annealing temperature of 55 °C and 23 cycles for both Gli and ptc and 16 cycles for actin was used (actin served as an internal control). The amplified products were 252 bp (Gli) and 453 bp (ptc). Both expression of Gli and ptc were found to be substantially exclusive to the mesodermal fraction of the yolk sac.

Example 6: Synergistic effect of Hedgehog protein with TGF-β proteins on hematopoiesis (and vascular growth)

Using the methods of Example 3(A) above, we have shown using RT-PCR, that both *Indian Hedgehog* and *BMP-6* are expressed in early visceral endoderm. Whole embryo (6.5dpc), epiblasts, epiblasts plus hedgehog protein, epiblasts plus BMP-6 protein and epiblasts plus hedgehog protein and BMP-6; are examined after 72 hrs incubation to determine the extent of activation of ϵ-globin expression. The experiment is repeated for BMP-2, BMP-4 and BMP-7. We expect to observe an enhanced effect when both hedgehog and BMP-4 are present compared with either alone.

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